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Effects of growth-promoters on the productivity of *Arundo donax* L. (NileFiber™) grown as a purpose-grown biofuel feedstock in Nova Scotia*

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Abstract: *Arundo donax* L. is a perennial grass species of Mediterranean origin. Its high biomass yield potential in its native climate has contributed to *A. donax* being an important biofuel feedstock crop. This study investigates the potential of *A. donax* as a purpose-grown biomass feedstock for advanced biofuel production in Nova Scotia. The effects of nine different growth-promoters (bacterial and fungal species, a seaweed extract, and a lipo-chitooligosaccharide) and two inoculation methods on *A. donax* biomass productivity were investigated through three experimental trials: one greenhouse and one field trial inoculated by a post-planting application of the supplements around the plant stem ("soil drenching") and one greenhouse trial with inoculation by root soaking just prior to planting (2016). The survival of *A. donax* over the winter was also evaluated over two winter seasons (2014-15 and 2015-16). *Penicillium bilaii* illustrated the most consistent positive effects on biomass productivity across experimental trials, and the plants' higher phosphorus concentrations suggest that *P. bilaii* was helping the plant access soil phosphorus. The root soaking inoculation method resulted in more positive effects in measured growth parameters compared with the soil drench method. Despite a very mild winter in one of the 2 years tested, *A. donax* did not successfully overwinter in the field in either year. The presence of growth-promoters did not enhance biomass productivity substantially in the field. Moreover, due to its low over-

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wintering survivability, this study suggests that the grass is not well suited as a biomass feedstock crop in Nova Scotia.

Short Title (Running Head): Peters et al. – *Arundo donax* as biofuel feedstock in Nova Scotia

Keywords: *Arundo donax*, agricultural biomass, biofuel, growth-promoter, lignocellulosic, purpose-grown

In Canada, the primary motivation for expanding the renewable energy industry is to reduce greenhouse gas emissions (Mabee and Saddler 2010) and the transportation sector, being a major contributor (Bhullar et al. 2012; Environment Canada 2013), is an area of particular focus. Bioenergy is often overlooked in renewable energy discussions, but liquid biofuels (bioethanol and biodiesel) are the most promising alternative to fossil fuels in the transportation sector.

First generation (1G) bioethanol is produced through fermentation from edible food and feed crops, most notably corn, wheat, soybean and sugarcane (Sims et al. 2010; Dutta et al. 2014). There are many drawbacks associated with 1G bioethanol production, including the food versus fuel debate (Serra and Zilberman 2013; Myers et al. 2014), dependence on non-renewable energy inputs (i.e. synthetic fertilizers, pesticides and agricultural equipment usage) (Hill et al. 2006) and often negligible reductions in greenhouse gas emissions (Bonin and Lal 2012).

Second generation (2G) bioethanol is produced from non-food biomass (i.e. lignocellulosic feedstock) (Naik et al. 2010; Demirbas 2011). Herbaceous energy crops, such as perennial grasses (*Panicum virgatum* and *Miscanthus* spp.) (Lewandowski et al. 2003) and short rotation coppice (SRC) including *Populus* spp. and *Salix* spp. combine to encompass purpose-grown feedstocks. Additionally, waste materials can also be sourced for biofuel: agricultural waste, including food crop residues (corn stover, wheat straw, etc.), forestry waste (by-products of conventional forest activities) (Tilman et al. 2009) and municipal solid waste (Demirbas 2008; Tilman et al. 2009). More complex biochemical or

thermochemical processes are utilized in 2G bioethanol production compared to the production of 1G bioethanol (Bessou et al. 2010).

Many drawbacks associated with 1G bioethanol production have been addressed through 2G bioethanol development (Naik et al. 2010; Wang et al. 2012). The capability of diversified biomass to be converted into 2G bioethanol reduces profound reliance on food crops as feedstocks (Sims et al. 2010). Lignocellulosic feedstock crops are mostly perennial species (Yuan et al. 2008; Cherubini et al. 2009) that have a higher nitrogen use efficiency than their annual counterparts, requiring less synthetic nitrogen fertilization and decreased risk of losing nitrogen to external sources (Don et al. 2012). Due to their perennial nature, lignocellulosic feedstock crops can increase long-term storage of soil carbon (Don et al. 2012; Wang et al. 2012).

Current Canadian bioethanol production is heavily dependent on food and feed crops (1G): in 2010, 91 % (~ 1.9 billion L) of Canadian-produced bioethanol was corn based, wheat based or corn-wheat mixture based (Scaife et al. 2015). Canadian 1G bioethanol production has slowly increased since 2008 (~ 1,800 million L in 2012) (Mabee 2013; Bradburn 2014) while bioethanol consumption based solely on national blending mandates (5 % bioethanol by volume in petroleum gasoline) is approximately 2,200 million L (Mabee 2013; Dessureault 2015).

Arundo donax L. is a C3 perennial rhizomatous grass (Corno et al. 2014; Pompeiano et al. 2015) of the *Poaceae* (Graminae) family (Polunin and Huxley 1966; Corno et al. 2014) and is the largest species of the *Arundo* genus (Perdue 1958; Bell 1997). *A. donax* thrives in Mediterranean conditions (Mardikis et al. 2001; Lambert et al. 2014) and originates from Asia (Polunin and Huxley 1966; Corno et al. 2014). *Arundo donax* was introduced to North America in the early 1800s as a form of erosion control in California, USA (Bell 1997; Ahmad et al. 2008; Mariani et al. 2010). Other global uses of *A. donax* include: leaf weaving for baskets and roofing material (Bell 1997; Mariani et al. 2010), aerial stems as musical instrument reeds (Perdue 1958; Fiore et al. 2014), non-wood paper production in Greece (Ververis et al. 2004) and natural reinforcement in polymer composites (as opposed to synthetic

reinforcements) (Fiore et al. 2014; Porras et al. 2016). More recently, *A. donax* has become a feedstock crop for liquid biofuels in Italy, serving as the main dedicated energy crop fueling Beta Renewable's commercial-scale lignocellulosic bioethanol plant in Crescentino (Palmqvist and Lidén 2014; Guo et al. 2015; Nogué and Karhumaa 2015). *Arundo donax* rarely produces viable seed, therefore reproduction is asexual through vegetative plant propagules including rhizomes, nodes and fragmented stems (Corno et al. 2014; Dragoni et al. 2015; Tauler and Baraza 2015). High *A. donax* biomass productivity has been noted in Mediterranean climates of Italy (37.7 – 38 t DM ha⁻¹) (Angelini et al. 2009; Mantineo et al. 2009). Certain frost-tolerant genotypes of *A. donax* are known to grow in cooler, more northern climates, such as Virginia (39 t DM ha⁻¹, Smith et al. 2015) and northeastern Oregon (Porter et al. 2012), both of the United States of America, as well as northeastern Germany (Bacher et al. 2001). To our knowledge, this is the first study of *A. donax* as a biomass feedstock in a climate as cool as Nova Scotia's (i.e. zone Dfb in the updated Köppen-Geiger climate classification system; Kottek et al. 2006).

Arundo donax is known to respond relatively poorly to agronomic inputs (Angelini et al. 2005). Given this, some have speculated that rhizospheric and/or endophytic plant growth-promoting microorganisms may play a role in sustaining yield on nutrient poor soils. Plant growth-promoters (GP) can be loosely defined in the context of this research as rhizospheric microorganisms and substances that are beneficial to plant growth under certain climatic and edaphic conditions (Vessey 2003; Bashan and de-Bashan 2005; Bhattacharyya and Jha 2012). Promoters can directly or indirectly promote plant growth through various relationships and mechanisms when in association with a host plant (Vessey 2003; Gray and Smith 2005; Glick 2012). Modes of action of growth-promoters include: biological nitrogen fixation (Gowariker et al. 2009; Ahemad and Kibret 2014), increasing soil nutrient availability (Bienfait 1989; Richardson et al. 2009; Shen et al. 2011) and production of phytohormones (auxins, cytokinins, gibberellins) (Patten and Glick 1996; Ma et al. 2011; Ha et al. 2012; Tanimoto 2012; Liu et al. 2013; Lugtenberg et al. 2013; Cassán et al. 2014).

Arundo donax, like many other candidate biomass feedstocks, produces high biomass yields on low agronomic inputs, substantiating its potential for biofuels. The addition of GP treatments can sustainably enhance overall biomass productivity, as well as other aspects of crop growth and development (i.e. photosynthesis, nitrogen fixation, etc.), as studied in biomass feedstocks (Ker et al. 2014; Wang et al. 2015; Fei et al. 2017). One of the GP treatments used in this study (a seaweed extract) has been shown to increase freezing/cold stress tolerance in *Arabidopsis thaliana* (Rayirath et al. 2009) and in *A. donax* (Zhao 2016). Various strains of *Gluconacetobacter* sp. used as GP treatments in this study have previously been shown to enhance growth and N₂ fixation in sugar beets grown as a biomass feedstock (Fei et al. 2017). Lipo-chitooligosaccharides (LCO), a treatment used alone and in combination with other GPs in this study, have been shown to enhance the early growth of C4 grasses (Tanaka et al. 2015).

This study investigated the effects of nine different plant GP and two application methods on biomass productivity of *A. donax* (NileFiber™). The objectives were: 1) to investigate the application of growth-promoter microorganisms and stimulants to enhance NileFiber™ biomass productivity, and 2) to evaluate the growth potential and winter survival of NileFiber™ for use as a purpose-grown advanced biofuel feedstock crop in marginal soils in Nova Scotia.

MATERIALS AND METHODS

Initial Planting – 2014

NileFiber™ rhizomes (NileFiber™ US 20140075628 P1 patent pending by TreeFree Biomass Solutions Inc. of Seattle, WA) were planted, per NileFiber Atlantic Canada Inc. recommendations, at a depth of 20 cm using an apple tree planter at the experimental field site at the Nappan Research Farm of Agriculture & Agri-Food Canada in Nappan, Nova Scotia (latitude 45°46'N, longitude 64°14'W) in July 2014. Due to low emergence of NileFiber™, dormant buds were excised from nodes beneath the soil in December 2014 for in vitro tissue culture propagation to be used in further experimentation.

In vitro tissue culture propagation

In vitro tissue culture propagation was performed following modified protocols (Cavallaro et al. 2011; Cavallaro et al. 2014). Buds were sterilized by rinsing with cold tap water, soaking in 70 % (v/v) bioethanol for one minute, further soaking in 20 % Javex® (1 % available sodium hypochlorite) for twenty minutes and were finally rinsed with autoclaved distilled water. The propagation procedure consists of three different phases in a growth chamber based on tissue culture growth: a) shoot growth, b) rooting (plantlet generation) and c) shoot proliferation. Varying constituents were added to the basal medium depending on tissue culture growth phase. Upon attaining a sufficient plant population for further experimentation, propagation was terminated.

GP treatment preparation

GP treatments used included in-lab cultivated bacteria and commercially available plant stimulants (Table 1). Two bacterial strains (*Azospirillum brasilense* N8 and *Variovorax paradoxus* JM63) were cultured using lysogeny broth (LB) liquid medium (Bertani 1951) while the remaining bacterial strains were cultured using modified LGI-P medium (Cavalcante and Döbereiner 1988; Pan and Vessey 2001). The LB medium consisted of (quantities per litre): 10 g tryptone; 5 g yeast extract; 10 g NaCl. The modified LGI-P medium consisted of (quantities per litre): 0.2 g K₂HPO₄; 0.6 g KH₂PO₄; 0.2 g MgSO₄ •

7H₂O; 0.02 g CaCl₂ • 2H₂O; 0.002 g NaMoO₄ • 2H₂O; 0.01 g FeCl₃ • 6H₂O; 5 mL bromothymol blue solution in 0.2 M KOH; 0.0001 g Biotin; 0.0002 g Pyridoxal HCl; 100 g sucrose; 1.32 g (NH₄)₂SO₄. Both media were autoclaved for fifteen minutes at 121.1 °C. Upon cooling, 1 mL of each bacterial suspension of *A. brasilense* N8 and *V. paradoxus* JM63 were added to the autoclaved LB medium and 0.5 mL of each other bacterial suspension was added to the autoclaved LGI-P medium. All media were placed in an orbital shaker at 125 rpm and 30 °C, the LB bacteria was cultured for three days and the LGI-P bacteria was cultured for four days.

Plantlet Acclimatization

Upon completion of the plantlet generation (rooting) stage of tissue culture propagation, NileFiber™ plantlets were transferred from the growth chamber to the Saint Mary's University greenhouse in Halifax, Nova Scotia (latitude 44°39'N, longitude 63°35'W). Plantlets were removed from culture boxes, rinsed thoroughly with cold tap water (to remove residual medium) and placed in a cold tap water bath for transport to the greenhouse. Plantlets were transplanted into plastic plant cell packs (one plantlet per cell) and filled with Pro-Mix® HP Mycorrhizae™. Cell packs were covered with clear plastic domes to increase humidity then placed in seed trays without drainage. The plastic domes were shifted off and eventually removed to gradually reduce humidity experienced by plantlets. Plantlets were moved outside the greenhouse as a final phase of acclimatization.

Experiment 1 – Greenhouse Root Soaking Trial

Fifteen GP treatments were applied to individual NileFiber™ plantlets through root soaking immediately after being removed from the growth chamber (15 replicates). Root inoculation of bacterial and fungal GPs is common agricultural practice. Although foliar sprays of *A. nodosum* extract is the most common application method, positive effects of root drenching applications have also been reported (Abkhoo and Sabbagh, 2016; Ali et al. 2016). Plantlets were suspended in an inoculum broth containing the GP treatment (Table 1) and phosphate buffer (34 mM, pH = 6.0) for thirty minutes (the *P. bilaii*

inoculum contained distilled water rather than phosphate buffer and contained 0.1 % Tween®20 to stabilize the emulsion of fungal spores). Single plantlets (10 – 15 cm height from soil surface) were transplanted into 0.8 L square pots (7 cm depth) and filled with 130 g of sterilized PREMIER® Top Soil (soil was sterilized for five hours at 200 °C using an electric soil sterilizer). The concentration of the GP treatments was 10^8 colony forming units (CFU). The concentration of the *A. nodosum* treatment was 1 mL L⁻¹. The concentration of LCO in the combinatory treatments was 1.8 % LCO (~ 100 µL plant⁻¹). Plantlets suspended in *P. bilaii* and *A. nodosum* received 5 mL plant⁻¹ inoculum through soil drench upon planting. The remaining plants received 5 mL plant⁻¹ phosphate buffer through soil drench.

Experiment 2 – Greenhouse Soil Drench Trial

Nine GP treatments were applied to individual NileFiber™ plantlets through soil drenching (i.e. applying solution, suspension or broth of the GP around the base of the plantlet) thirty-five days after transplantation from the growth chamber to the greenhouse. Single plantlets (30 – 35 cm in height above the soil surface) were transplanted into 3 L pots (7 cm depth) filled with 2 kg of PREMIER® Top Soil (Fig. 1a.). Each GP treatment contained 5×10^8 CFU of bacteria (or fungal spores, *P. bilaii*) diluted in 45 mL of water (total volume = 50 mL). The concentration of the *A. nodosum* treatment was 1 mL plant⁻¹ and the concentration of LCO in both the individual and combinatory treatments was 100 µL plant⁻¹. There were 12 replicates for each GP treatment.

Experiment 3 –Field Soil Drench Trial

Six GP treatments were applied to individual NileFiber™ plantlets through a soil drench application 70 days after transplantation out of the growth chamber (field planting, 15 June 2015; soil drench application, 10 July 2015). Single plantlets were planted (7 – 10 cm depth) into a coarse, loamy (Class 2) soil of the Nappan field site (slope ≈ 3%) (Webb and Langille 1995) (Fig. 1b.). GP treatments were arranged in a randomized complete block design with five plot replicates per treatment. Plant density was 6.25 plants m⁻², row distance was 50 cm and there was a buffer zone of 1 m between plots. Each GP

treatment contained 5×10^8 CFU of bacteria (or fungal spores, (*P. bilaii*)) diluted in 245 mL of water (total volume = 250 mL). The concentration of the *A. nodosum* treatment was 1 mL L⁻¹ plant⁻¹. Plant crowns and rhizomes from Experiment 3 remained in the field during the 2015-16 winter season to assess winter survival the following spring.

Data Collection

Growth parameters were measured at harvest, approximately four months after GP treatment application in Experiments 2 and 3 and approximately two months after GP treatment application in Experiment 1. Growth parameters measured include plant height, shoots per plant, above-ground biomass fresh and dry weight, and root dry weight. Plant height (soil surface to the highest collar region on the main stem) and shoots per plant (height > 5 cm) were collected prior to cutting. Above-ground biomass fresh weight was measured immediately after collection. Roots were unearthed from pots, soil was gently shaken off and roots were thoroughly rinsed with water. Roots and above-ground biomass (from greenhouse trials) were dried at 80 °C for eight days and corresponding dry weights were subsequently measured.

Statistical Analyses

Data were analyzed by two statistical approaches to assess differences in measured growth parameters between GP treatments: 1) frequentist approach (one-way ANOVA) and 2) Bayesian approach (Metric Predicted Variable with One Nominal Predicted Variable). When the F-statistic from the ANOVA was significant, treatment means were separated by the least significance difference (LSD) test ($P < 0.05$) (Table 2). For the Bayesian approach, a t-distribution with non-informative prior probabilities was used as the prior distribution. The Markov Chain Monte Carlo (MCMC) method was used to generate posterior distributions. All statistical analyses were completed using RStudio Version 0.99.484.

RESULTS

Initial Planting – 2014

Only 30% of the rhizomes of NileFiber™ planted in 2014 successfully emerged. These plants grew over the 2014 growing season and were left in the field over the winter of 2014-15. No new growth emerged in the spring of 2015, indicating zero winter survival.

Experiment 1 – Greenhouse Root Soaking Trial

The GP treatments affected each of the three measured growth parameters (shoots per plant, above-ground biomass fresh weight per plant (FW) and above-ground biomass dry weight per plant (DW)) in a similar pattern (Fig. 2c; Table 2). For example, the S treatment always resulted in the lowest growth responses, and the A treatment always invoked the greatest responses. Three combinatory treatments (AL, DL and VPL) as well as treatments D and PL exhibited significantly greater number of shoots per plant than the untreated control ($P < 0.01$). All combinatory GP treatments except GSL had higher values than the Bayesian estimated number of shoots per plant (2.7903 shoots) while the control treatment had a lower value. There were no credible non-zero differences between treatments.

Four combinatory treatments (AL, DL, GL and VPL) as well as four other treatments (G, GS, PB and VP) exhibited significantly greater FWs than the untreated control ($P < 0.0001$). Three of the four combinatory GP treatments also exhibited greater FWs than their individual counterparts (AL, DL and VPL) with AL and DL weighing significantly greater than A and D, respectively. The S treatment was the only treatment to show a significantly lower FW than the untreated control (Fig. 2b; Table 2). Four combinatory treatments (AL, DL, GPL and VPL) in addition to four individual treatments (G, GS, PB and VP) showed a credible positive effect from the Bayesian estimated mean (FW = 3.3532 g) and the remaining seven treatments showed a credible negative effect. Four treatments (AL, D, GSL and S) showed credible non-zero differences, with AL showing a credible positive non-zero difference and S showing a credible negative non-zero difference when compared with the untreated control.

Five of the six combinatory treatments (all but GSL) and seven individual GP treatments showed significantly greater DWs than the untreated control ($P < 0.0001$). Combinatory treatment GSL exhibited lesser DW than its individual counterpart (Fig. 2a; Table 2). Four combinatory treatments (AL, DL, PLL and VPL), in addition to six individual treatments (AB, D, GP, GS, PB and VP) showed a credible positive effect from the Bayesian estimated mean (DW = 1.0072 g) and the remaining five treatments showed a credible negative effect. Five treatments (AL, GSL, S, VP and VPL) showed credible non-zero differences, with treatments AL, VP and VPL exhibiting a credible non-zero difference when compared to the untreated control.

Experiment 2 – Greenhouse Soil Drench Trial

NileFiber™ height and above-ground biomass fresh weight (FW) were negatively affected by the presence of three of the four LCO treatments (AL, GL, L) while root dry weight (RDW) was negatively effected by all four LCO treatments. Plants treated with treatments AL, GL and L had significantly lower heights ($P < 0.01$) (Fig. 3c) and FWs ($P < 0.01$) (Fig. 3b) than untreated control plants (Table 2). These treatments also exhibited credible negative effects from the Bayesian estimated means (height = 112.99 cm, FW = 43.13 g). NileFiber™ RDWs for all combinatory treatments (AL, GL, L and PBL) were significantly lower ($P < 0.001$) (Fig. 3a; Table 2) and exhibited credible negative effects from the Bayesian mean estimate (RDW = 3.6 g). Treatments PB and PBL exhibited statistically similar measurements to the untreated control treatment and credible positive effects (compared to Bayesian estimated means) in height and FW.

Experiment 3 – Field Soil Drench Trial

Shoots per plant was the only measured growth parameter to exhibit statistically important results in the field experiment. Plants treated with PB and D exhibited the same number of shoots per plant as the untreated control plants ($P < 0.05$) (Fig. 4; Table 2) and a credible positive effect from the Bayesian estimated mean (8.181 shoots). Plants treated with treatments A, G and S exhibited significantly less

shoots per plant than the control; in addition, treatments A and G showing credible non-zero differences compared to the untreated control. After the winter season of 2015-16, there was no NileFiber™ survival at the field site.

DISCUSSION

Initial Planting (2014) and Winter Survival (2014-15 and 2015-16)

The initial planting of NileFiber™ at the field site was deemed unsuccessful based on low emergence (~ 30 %) in the establishment year. There are various potential reasons for the low emergence and unsuccessful establishment of NileFiber™ after the 2014 planting including 1) timing of planting and 2) seasonal temperature variation. NileFiber™ rhizome fragments were planted at the end of July, which is late into the growing season: the recommendation for *A. donax* planting to increase biomass yield is early spring (Copani et al. 2013; Cavallaro et al. 2014).

Hardiness zones are defined by climate variables and the relative probability of successful survival of a plant species based on those variables (Ouellet and Sherk 1967; McKenney et al. 2001; McKenney et al. 2006). Since the introduction of the original Canadian hardiness zones in 1967 by Ouellet and Sherk, these models have been modified to consolidate the systems used by the United States Department of Agriculture (USDA) and Agriculture & Agri-Food Canada (AAFC) by incorporating extreme minimum temperature (McKenney et al. 2006). Through this consolidation, the Canadian Plant Hardiness zones can be compared to numerous other countries whose systems are also based around the USDA system.

Arundo donax, a species that thrives in a Mediterranean climate (Lambert et al. 2014) is also used as a purpose-grown feedstock for bioethanol in Vercelli, Italy (Bomgardner 2013; Palmqvist and Lidén 2014). Based on a similar system to the USDA Plant Hardiness Zone system (extreme minimum temperature), the location of the bioethanol plant falls into Zone 8b (-9.4 to -6.7 °C) (PlantMaps 2016a). In the southern United States, where *A. donax* has been introduced and in some states, has become invasive, these areas fall into Zones 8 through 10 (-12.2 to 4.4 °C) (United States Department of Agriculture

(USDA) 2012a; USDA 2016). Further north, in Boardman, Oregon (Porter et al. 2012) (hardiness Zone 7b (-15 to -12.2 °C) (PlantMaps 2017a)) and in Blacksburg, Virginia (Smith et al. 2015) (Zone 6b (-20.6 to -17.8 °C) (PlantMaps 2017b)), *A. donax* has been successfully cultivated as a biomass feedstock. When classified using the extreme minimum temperature approach, Nova Scotia falls into Zones 5 through 7a (-28.9 to -15.0 °C) (Natural Resources Canada (NRC) 2014a; NRC 2014b; PlantMaps 2016b) (Fig. 5).

During the 2014 – 15 winter season (after the Initial Planting), the field site experienced below average temperatures while in the 2015 – 16 winter season (after Experiment 3), the field site experienced above average temperatures (Fig. 6). Despite this variation in temperature over the two winter seasons, NileFiber™ did not survive either of the two winter seasons. Nova Scotia winter temperatures in 2014-15 were more characteristic of Zone 3a (i.e. Fort McMurray, AB) than Zones 5 through 7 (Environment Canada 2015a). Although Nova Scotia experiences more extreme minimum temperatures than areas where *A. donax* establishes, in theory *A. donax* could survive in average Nova Scotia climatic conditions. An estimated 2% of Canada is suitable for *A. donax* establishment including parts of Nova Scotia, based on successful establishment in Plant Hardiness Zones greater than Zone 6 (USDA 2012b). However, in the current study, NileFiber™ did not even survive the warmer of the two winter seasons at the Nappan field site.

Experiment 1 – Greenhouse Root Soaking Trial

NileFiber™ plants treated with the combinatory AL treatment had a positive impact on measured growth parameters compared to the control treatment while the S treatment showed negative impacts.

The positive effect of the combinatory treatments, namely treatments AL, DL and VPL on NileFiber™ could be attributed to an interactive effect between the bacteria and the LCO. The PB treatment exhibited similar positive effects as the AL treatment, which could be indicative that phosphorus is the macronutrient most lacking in these edaphic conditions. *Penicillium bilaii* is reported to mobilize plant unavailable phosphorus (Wakelin et al., 2007; Shen et al., 2011). *Arundo donax* is also

known to exhibit high phosphorus uptake and accumulation (Sagehashi et al. 2009; Kering et al. 2012). The negative effect of the S treatment on NileFiber™ could be attributed to the relatively high concentration of extract coming in direct contact with the root systems for 30 minutes. Although positive effects of root drench applications of *A. nodosum* extract have been reported (Abkhoo and Sabbagh, 2016; Ali et al. 2016), most liquid seaweed extract applications are applied foliarly, and higher concentrations have shown inhibitory effects (Khan et al. 2009; Craigie 2011). Also, in preliminary experiments in our laboratory, poplar cuttings showed greater adventitious root development by soaking in low concentrations of *A. nodosum* extract rather than at higher concentrations (data unpublished).

Experiment 2 – Greenhouse Soil Drench Trial

There was little positive impact of GP treatments on NileFiber™ growth compared to the control. The GL treatment showed an overwhelmingly negative trend in FW, DW and RDW compared to the control. In addition, the other L treatments (AL & L) also exhibited negative effects on plant growth. The PBL treatment exhibited similar trends in effects on plant growth as the control treatment.

The negative effects of LCO treatments on NileFiber™ growth may have occurred because of prior colonization of the plant rhizosphere. If NileFiber™ had already formed a relationship with rhizospheric organisms, these organisms may have identified the exogenously applied LCO as a pathogenic substance, and thereby interfered with the rhizospheric organisms' interactions with the host plant. Alternatively, the exogenous LCO may not have been identified at all: in this experiment, LCO treatments were diluted with distilled water, giving a final concentration of 10^{-12} M LCO where the most commonly studied concentration with growth-promoting activity is between 10^{-6} – 10^{-8} M (Prithiviraj et al. 2000; Smith et al. 2005; Schwinghamer et al. 2016).

The PBL treatment exhibited effects on plant growth unlike the other LCO treatments, more similar to the positive effect of the control treatment. TagTeam®LCO (Novozymes®) is a commercial inoculant that combines *P. bilaii* and LCO with a rhizobium species to form a single inoculant to improve plant

growth. Firstly, as mentioned, *P. bilaii* increases microbial phosphorus through mobilization (Wakelin et al. 2007; Shen et al. 2011). Secondly, the combination of LCO and the rhizobia create the signalling cycle necessary to fix more nitrogen for plant use (Prithiviraj et al. 2003; Novozymes 2011). This inoculant exhibits plant growth-promoting effects based on the presence of the rhizobium: although the PBL treatment did not contain a bacterial species, there could have been a similar interactive process between the PBL treatment and some native soil bacteria, or the positive effects of this treatment could be based solely on the presence of *P. bilaii*.

Experiment 3 – Field Soil Drench Trial

The field experiment evaluated a) the performance of six GP treatments on NileFiber™ growth and biomass productivity and b) the survival of NileFiber™ through a Nova Scotian winter.

NileFiber™ plantlet density in the field was 6.25 plants m⁻² with dry biomass yields in the establishment year (2015) ranging from 1.68 – 1.85 Mg ha⁻¹. This planting density is comparable with other studies reporting much lower planting densities with increasing biomass yields in subsequent post-establishment harvests (Cosentino et al. 2006; Pari et al. 2016; Testa et al. 2016). Although comparable, future yields may decline with this relatively high planting density, as previous studies have shown that after the establishment season, lower planting densities prevailed with higher yields than higher planting densities (Angelini et al. 2005; Smith et al. 2015). *A. donax* L. has a reportedly long establishment time in ideal conditions, reaching stabilized dry matter yields three years post-planting (Lewandowski et al. 2003; Cavallaro et al. 2014; Cosentino et al. 2014) perhaps based on its perennial nature (Smith et al. 2015).

There is less variance between growth parameter measurements in the field compared with the greenhouse soil drench experiment, creating less distinction between treatments. NileFiber™ plants treated with the PB and D treatments showed similarities to control plants while the A and G treated plants had less shoots than the control. The positive effect of the PB treatment could be attributed to the

aforementioned action of *P. bilaii* creating phosphorus stores to support the high phosphorus uptake of *A. donax* (Sagehashi et al. 2009; Kering et al. 2012). Despite evidence supporting increased *Arundo donax* tolerance to cold stress in Nova Scotia after treatment with *Ascophyllum nodosum* extract (concentration 1 mL L⁻¹) (Zhao 2016), NileFiber™ did not overwinter in the climatic and edaphic conditions of the field site despite being well established in the 2015 growing season and experiencing a warmer than average winter season in 2015-16.

GP Application Method

Root colonization is the most basic yet important step in establishing a beneficial relationship between plants and promoters (Ahmad et al. 2011; Mangmang et al. 2015). The delayed soil drench treatment of NileFiber™ could have enabled native microorganisms to colonize root systems and establish their rhizospheric role prior to the supplemental exogenous GP application (Schipper et al. 1987; Requena et al. 1997). The root soaking application method (Experiment 1) was more conducive to plant-promoter interaction because the roots were in direct contact with the intended promoters for 30 minutes prior to planting, simplifying root colonization and virtually eliminating colonization of unintentional rhizospheric species. Additionally, in comparison to a foliar application, a root soaking GP application occurs at the same time as planting, lowering costs and creating a more sustainable operation. On a larger scale, pre-planting root soaking is neither efficient nor practical, hence the soil drench experiments (Experiments 2 & 3). Soil drenching (post-planting) mimics some current agricultural practices, but this application method but does not ensure contact between plant roots and the growth promoters.

Soil Properties

Sterilized soil was used in Experiment 1 to attempt to optimize root colonization by intended microbial GP treatments, but this is not agriculturally realistic as soil sterilization is a highly intensive procedure in terms of time, energy and resources. Sterilized soil can promote greater colonization of the

host plant by GPs due to less microfloral competition: there was a greater *A. brasilense* concentration in axenic systems of wheat (*Triticum aestivum* L.) as compared to natural soil (Schloter et al. 1998). Sterile conditions may also allow for a potentially inhibitory GP concentration as the GP treatment would be more directly available to plants due to lower competition (no naturally occurring microorganisms or substances) (Requena et al. 1997). Experiments 2 and 3 were conducted with non-sterile soil, creating more diverse rhizospheric situations in which the intended promoters may be less effective or may combine with naturally occurring microorganisms or substances to increase effectiveness.

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CONCLUSION

This study is the first testing of the growth potential of *A. donax* in a climate as cool as Nova Scotia. The growth-promoter treatments indicated that NileFiber™ growth is not greatly influenced by the presence or absence of GPs in the field. Under greenhouse conditions, GP application through root soaking (at planting) can enhance *A. donax* productivity, but such an inoculation method is not a viable option on a large scale. Given the poor winter survival of *A. donax* in the field over two consecutive winters in Nova Scotia, biomass producers in climates similar to Nova Scotia's may wish to investigate other potential lignocellulosic (2G) biomass feedstock crops (e.g. *Panicum virgatum* L., and *Miscanthus* sp.).

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Abbreviations: **1G**, first generation; **2G**, second generation; **A**, *Azospirillum brasilense* N8; **AL**, *A. brasilense* N8 + LCO; **ANOVA**, analysis of variance; **C**, Untreated control; **D**, *Gluconacetobacter azotocaptans* DS1; **DL**, *G. azotocaptans* DS1 + LCO; **DW**, above-ground biomass dry weight; **FW**, above-ground biomass fresh weight; **G**, *Gluconacetobacter diazotrophicus* PAL5T; **GL**, *G. diazotrophicus* PAL5T + LCO; **GP**, growth-promoter; **GS**, *Gluconacetobacter diazotrophicus* SRT4; **GSL**, *G. diazotrophicus* SRT4 + LCO; **L**, lipo-chitoooligosaccharide; **LB**, lysogeny broth; **LCO**, lipo-chitoooligosaccharide; **LSD**, least significant difference; **MCMC**, Markov Chain Monte Carlo; **PB**, *Penicillium bilaii*; **PBL**, *P. bilaii* + LCO; **PL**, *Gluconacetobacter diazotrophicus* PAL5T Lsd B++; **PLL**,

G. diazotrophicus PAL5T Lsd B++; **RDW**, root dry weight; **S**, *Ascophyllum nodosum*; **SRC**, short rotation coppice; **VP**, *Variovorax paradoxus* JM63; **VPL**, *V. paradoxus* JM63 + LCO.

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Fig. 1. *Arundo donax* L. (NileFiber™) in the greenhouse during Experiment 2 (Greenhouse Soil Drench) (A) and plants from Experiment 3 (Field Soil Drench) being harvested at the field site in Nappan, Nova Scotia in October 2015 (B).

Fig. 2. Mean above-ground biomass dry weight (g) (A), fresh weight (g) (B) and mean number of shoots (C) per NileFiber™ plant from experiment one. Treatments: C = Control; Individual: A = *A. brasilense* N8; D = *G. azotocaptans* DS1; G = *G. diazotrophicus* PAL5T; GS = *G. diazotrophicus* SRT4; PB = *P. bilaii*; PL = *G. diazotrophicus* PAL5T LsdB++; S = *A. nodosum*; VP = *V. paradoxus* JM63; Combination: AL = *A. brasilense* N8 + LCO; DL = *G. azotocaptans* DS1 + LCO; GL = *G. diazotrophicus* PAL5T + LCO; GSL = *G. diazotrophicus* SRT4 + LCO; PLL = *G. diazotrophicus* PAL5T LsdB++ + LCO; VPL = *V. paradoxus* JM63 + LCO. Different letters indicate significant difference at $P < 0.05$. Bars indicate standard error. (n = 15).

Fig. 3. Mean height (cm) (A), above-ground biomass fresh weight (g) (B) and root dry weight (g) (C) per NileFiber™ plant from experiment two. Treatments: C = Control; Individual: A = *Azospirillum brasilense* N8; G = *Gluconacetobacter diazotrophicus* PAL5T; L = lipo-chitoooligosaccharide; PB = *Penicillium bilaii*; S = *Ascophyllum nodosum*; Combination: AL = *A. brasilense* N8 + LCO; GL = *G. diazotrophicus* PAL5T + LCO; PBL = *P. bilaii* + LCO. Different letters indicate significant difference at $P < 0.05$. Bars indicate standard error. (n = 12).

Fig. 4. Mean number of shoots per NileFiber™ plant from experiment three. C = Control; A = *Azospirillum brasilense* N8; D = *Gluconacetobacter azotocaptans* DS1; G = *Gluconacetobacter diazotrophicus* PAL5T; PB = *Penicillium bilaii*; S = *Ascophyllum nodosum*. Different letters indicate significant difference at $P < 0.05$. Bars indicate standard error. (n = 45).

Fig. 5. Plant hardiness zones associated with successful field trials using *A. donax* (Porter et al. 2012; USDA 2012a; Bomgardner 2013; NRC 2014a; Smith et al. 2015; PlantMaps 2016a; PlantMaps 2016b; USDA 2016; PlantMaps 2017a; PlantMaps 2017b).

Fig. 6. Mean monthly field temperatures (°C) at Nappan, NS in 2015 and 2016 compared to the mean temperature (°C) from 1981 – 2010 (Environment Canada 2016*a*; Environment Canada 2016*b*).

For Review Only

177x82mm (300 x 300 DPI)

88x146mm (300 x 300 DPI)

88x146mm (300 x 300 DPI)

155x120mm (300 x 300 DPI)

Only

Fig. 5. Plant hardiness zones associated with successful field trials using *A. donax* (Porter et al. 2012; USDA 2012a; Bomgardner 2013; NRC 2014a; Smith et al. 2015; PlantMaps 2016a; PlantMaps 2016b; USDA 2016; PlantMaps 2017a; PlantMaps 2017b).

146x91mm (300 x 300 DPI)

184x165mm (300 x 300 DPI)

Table 1. Treatment names and abbreviations of GP treatments (individual and combination) applied to NileFiber™ in three separate experiments.

Treatment	Abbreviation	Exp.1	Exp.2	Exp.3
INDIVIDUAL				
<i>Ascophyllum nodosum</i>	S	✓	✓	✓
<i>Azospirillum brasilense</i> N8	A	✓	✓	✓
<i>Gluconacetobacter azotocaptans</i> DS1	D	✓		✓
<i>Gluconacetobacter diazotrophicus</i> PAL5T	G	✓	✓	✓
<i>Gluconacetobacter diazotrophicus</i> PAL5T Lsd B++	PL	✓		
<i>Gluconacetobacter diazotrophicus</i> SRT4	GS	✓		
Lipo-chitooligosaccharide (LCO)	L		✓	
<i>Penicillium bilaii</i>	PB	✓	✓	✓
<i>Variovorax paradoxus</i> JM63	VP	✓		
Untreated Control	C	✓	✓	✓
COMBINATION				
<i>A. brasilense</i> N8 + LCO	AL	✓	✓	
<i>G. azotocaptans</i> DS1 + LCO	DL	✓		
<i>G. diazotrophicus</i> PAL5T + LCO	GL	✓	✓	
<i>G. diazotrophicus</i> PAL5T Lsd B++ + LCO	PLL	✓		
<i>G. diazotrophicus</i> SRT4 + LCO	GSL	✓		
<i>P. bilaii</i> + LCO	PBL		✓	
<i>V. paradoxus</i> JM63 + LCO	VPL	✓		

Table 2. Mean growth parameters with Frequentist analysis (one-way ANOVA) results reported for experiments 1 through 3 for *Arundo donax* L. (NileFiber™) plants applied with PGPR treatments.

PGPR Treatment ^a	Experiment 1			Experiment 2			Experiment 3
	Shoots per Plant	FW (g)	DW (g)	Plant Height (cm)	FW (g)	RDW (g)	Shoots per Plant
<i>Individual</i>							
A	2.80abcd	3.20bcd	1.06abc	115.6ab	45ab	4.00ab	7.5b
D	3.07ab	2.87d	1.00bc	-	-	-	9.0a
G	2.67bcd	3.63ab	1.11ab	115.2ab	45ab	3.56bcd	7.8b
GS	2.40d	3.57abc	1.02bc	-	-	-	-
L	-	-	-	107.6bc	42bcd	3.44cde	-
PB	2.80abcd	3.73ab	1.07abc	114.5ab	43abc	3.85abc	8.4ab
PL	3.07ab	3.03cd	0.94cde	-	-	-	-
S	2.40d	2.30e	0.73f	115.7ab	42bcd	3.75abc	8.0b
VP	2.87abcd	3.63ab	1.13ab	-	-	-	-
C	2.47cd	3.00d	0.81ef	118.8a	47a	4.11a	9.3a
<i>Combination</i>							
AL	3.27a	4.07a	1.23a	102.6c	39cd	3.10de	-
DL	3.00ab	3.63ab	1.10abc	-	-	-	-
GL	2.87abcd	3.60ab	0.99bcd	107.8bc	38d	3.07e	-
GSL	2.73bcd	2.90d	0.84def	-	-	-	-
PBL	-	-	-	119.2a	43abc	3.60bc	-
PLL	2.93abc	3.37bcd	1.03bc	-	-	-	-
VPL	3.00ab	3.73ab	1.13ab	-	-	-	-

Note: Means followed by the same letter within a column are not significantly different at $P = 0.05$.

^aTreatment abbreviations are defined in Table 1.